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Inhibition of nitric oxide synthase accentuates endotoxin-induced sickness behavior in mice

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ABSTRACT

Sickness behavior appears to be the expression of a central motivational state that reorganizes an organism's priorities to cope with infectious pathogens. To evaluate the possible participation of nitric oxide (NO) in lipopolysaccharide-induced sickness behaviors, mice were submitted to the forced swim test (FST), open field test and dark–light box test. Food intake and corticosterone plasma levels were evaluated. Lipopolysaccharide (LPS, 100 μg/kg; i.p.) administration increased the time spent floating in the FST and decreased locomotor activity in the open field. Indeed, treatment with LPS decreased the total number of transitions between the dark and light compartments of the apparatus. In addition, LPS reduced food intake and increased corticosterone levels. Pretreatment with L-NAME (30 mg/kg; i.p.) or aminoguanidine (50 mg/kg; i.p.) accentuated the behavioral changes induced by LPS in the FST, open field and light–dark box tests as well as induced an increment in hypophagia and in corticosterone levels. These findings confirm previous observations that have reported LPS-induced sickness behaviors. In addition, they provide evidence that the synthesis of NO modulates changes in depressive-like and exploratory behaviors in mice, which is supported by the fact that NO synthase inhibitors also attenuate LPS-induced behavioral changes. In addition, the present study suggests that NO may have a protective role, acting in an inhibitory feedback manner to limit LPS-induced sickness behavior.

1. Introduction

Sickness behavior is an expression of a motivational state triggered by activation of the peripheral innate immune system whereby an organism reorganizes its priorities to fight infection (Parnet et al., 2002; Dantzer et al., 2008; Dantzer, 2009). Sickness behavior is easily induced in laboratory animals by the administration of endotoxins, which are the main active components of the outer membrane of gram-negative bacteria. Peripheral administration of endotoxin activates the immune system, resulting in the release of pro-inflammatory cytokines and prostaglandins (Long et al., 1990; Kelley et al., 2003; Engblom et al., 2002; Rorato et al., 2009; de Paiva et al., 2010). These peripherally produced cytokines gain access to the brain and produce the classical symptoms of sickness behavior, including reduction in locomotor activity and exploratory behaviors, anorexia and anhedonia (Engblom et al., 2002; Frenois et al., 2007). In addition, it has been demonstrated that upon stimulation by endotoxin and cytokines, nitric oxide synthase (NOS) becomes expressed, leading to the production of large amounts of nitric oxide (NO) in peripheral tissues and in the brain (Moncada et al., 1991; Giusti-Paiva et al., 2005; Mollace et al., 2005).

NO is produced from L-arginine by three different isoforms of NOS, two of which are expressed constitutively, endothelial (eNOS) and neuronal (nNOS) (Mollace et al., 2005). Endotoxin induces the expression of another calcium-independent inducible isoform of NOS (iNOS) in a number of tissues including the brain (Moncada et al., 1991; Mollace et al., 2005; Cauwels, 2007). Pharmacological inhibition of NOS can be used to elucidate the contribution of NO, and previous reports support both a detrimental and beneficial role of NO during immune challenges. An increase in the amount of NO produced by iNOS might play a pivotal role in the pathophysiology of sepsis (Thiemermann, 1997; Szabo, 1996; Vincent et al., 2000; Cauwels, 2007).

Although a significant volume of literature indicates that cytokines and prostaglandins are important mediators of sickness behavior (Dunn and Swiergiel, 2005; Dantzer et al., 2008; de Paiva et al., 2010), there is a lack of data on the role of NO in endotoxin-induced sickness behavior. To evaluate the possible participation of NO in LPS-induced sickness behaviors, mice were submitted to well-accepted tests to evaluate depressive-like and exploratory behaviors, including the forced swim test (FST), open field test and dark–light box test.

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2. Materials and methods

2.1. Animals

Adult male Swiss mice (22–30 g) were obtained from the Central Animal Facility of the Federal University of Alfenas. Animals were housed under controlled light (12:12 h light–dark cycle; lights on at 06:00 am) and temperature conditions (23 ± 1 °C) with access to water and food *ad libitum*. Animals were allowed to habituate to the housing facilities for at least 1 week before the experiments began. Behavioral studies were conducted in a quiet room between 09:00 and 11:00 a.m. to avoid circadian variation. All experiments were conducted in accordance with the Declaration of Helsinki addressing the welfare of experimental animals and with the approval of the Ethics Committee of the Federal University of Alfenas (protocol #0178/2008).

2.2. Experimental procedures

In the animal room, the mice were pretreated with non-selective NOS inhibitor (Nϖ-L-nitro-arginine methyl ester; L-NAME, 30 mg/kg), or a relatively selective iNOS inhibitor (aminoguanidine, 50 mg/kg), or vehicle (0.9% NaCl) 30 min before injections of lipopolysaccharide (LPS) serotype 026:B6 (100 μg/kg; i.p.) or saline (0.9% NaCl). The behavioral tests were performed 120 min after the LPS treatment. These time points were chosen on the basis of previous behavioral, endocrine and neurochemical studies (Dunn and Swiergiel, 2005; Rorato et al., 2009; de Paiva et al., 2010). The doses of L-NAME and aminoguanidine used in the present study are in agreement with the doses commonly used in other reports that injected these compounds peripherally (Giusti-Paiva et al., 2004). All drugs were purchased from Sigma-Aldrich Co. and dissolved in sterile isotonic saline.

2.2.1. Forced swim test

This test was performed according to the method developed by Porsolt et al. for mice. Mice (n = 12 per group) were placed in a vertical glass cylinder (26 cm high, 12 cm diameter) filled with 25 °C water to a depth of 16 cm. Water depth was chosen to ensure that animals swam or floated without their hind limbs or tail touching the bottom. Each mouse was placed in the cylinder for 6 min, and the duration of floating (i.e., the time during which mice made only the smallest movements necessary to keep their heads above water) was scored. As suggested by Porsolt et al. (1977), only the data scored during the last 4 min were analyzed and presented (Porsolt et al., 1977; Dunn and Swiergiel, 2005; de Paiva et al., 2010).

2.2.2. Open field behavioral test

Locomotor activity was quantified for 5 min in an open field box, consisting of white Plexiglas 60 × 60 cm in diameter with a floor divided into 16 squares. Previous studies have indicated that this time-period was sufficient to produce differences between treatment groups. Furthermore, after 5 min, the mice habituate to the apparatus, thereby decreasing the differences between groups. Four squares were defined as the center and the 12 squares along the walls were considered the periphery. Each mouse (n = 10 per group) was gently placed in the exact center of the box. Activity was scored as a line crossing when a mouse removed all four paws from one square and entered another. Line crossings among the central four squares or among the peripheral 12 squares of the open field were counted separately (Dunn and Swiergiel, 2005; de Paiva et al., 2010).

2.2.3. Light–dark box test

The apparatus consisted of a Plexiglas rectangular box (48 cm long × 24 cm wide × 24 cm high) divided into a dark region (24 cm long) and a light region (24 cm long). The light and dark regions were separated by an opening (8.0 × 8.0 cm) that allowed the animals to move between the two compartments. The dark region was made of black Plexiglas and covered with a black lid. The light portion was made of white Plexiglas, and a 60 W light was positioned directly over it. On the day of testing, each mouse was transported individually from the housing room to the testing room. Each mouse (n = 10 per group) was placed in the light compartment and allowed to move freely between the two compartments. The behavior was video-recorded for a total of 5 min, and the videotapes were scored for latency to the first transition and the number of transitions between the light and dark compartments (Lacosta et al., 1999; de Paiva et al., 2010).

2.2.4. Feeding behavior

The animals (n = 10 per group) fasted for 12 h before receiving injections. Immediately after injections, a fresh supply of pre-weighed food was given. Food intake was calculated at 2, 4, 6 and 24 h after the injection by measuring the difference between the pre-weighed standard chow available and the weight of chow and spilled crumbs at each time point. Changes in body weight were measured by weighing the animals at the beginning of the experiment as well as before and after an experimental day (Rorato et al., 2009; Soncini et al., 2012).

2.2.5. Plasma corticosterone assay

Trunk blood was collected from animals 2 h after LPS or saline injections in chilled heparinized tubes and was centrifuged at 3000 rpm for 15 min at 4 °C. Plasma was collected and stored at −20 °C. Plasma corticosterone was measured by radioimmunoassay.

2.2.6. Statistical analysis

The data obtained were analyzed using the GraphPad software program Version 4.0 and expressed as the mean ± S.E.M. Statistically significant differences among groups were calculated by the application of an analysis of variance (ANOVA) followed by the Newman–Keuls test. *P*-values less than 0.05 ($p<0.05$) were considered significant.

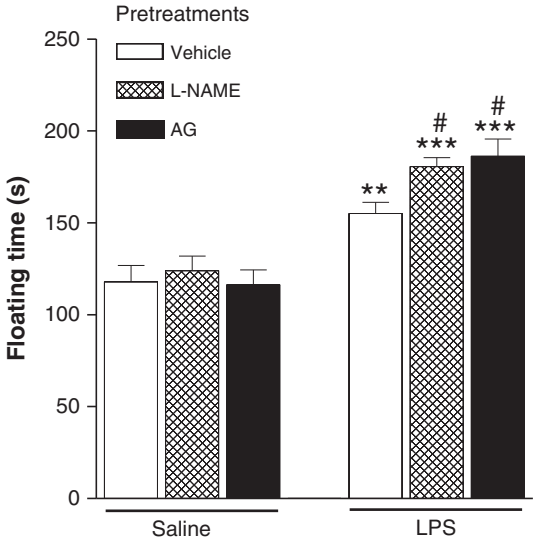

Fig. 1. Effects of pretreatment with vehicle, L-NAME (30 mg/kg) or aminoguanidine (AG, 50 mg/kg) on time spent floating in the forced swim test (n = 12 animals per group), which were measured 2 h after administration of either lipopolysaccharide (LPS) or saline. Each column represents the mean with S.E.M. The symbols denote significance levels: $^{**}p<0.01$; $^{***}p<0.001$ when compared with the control groups; # $p<0.05$ when compared with the vehicle plus LPS group.

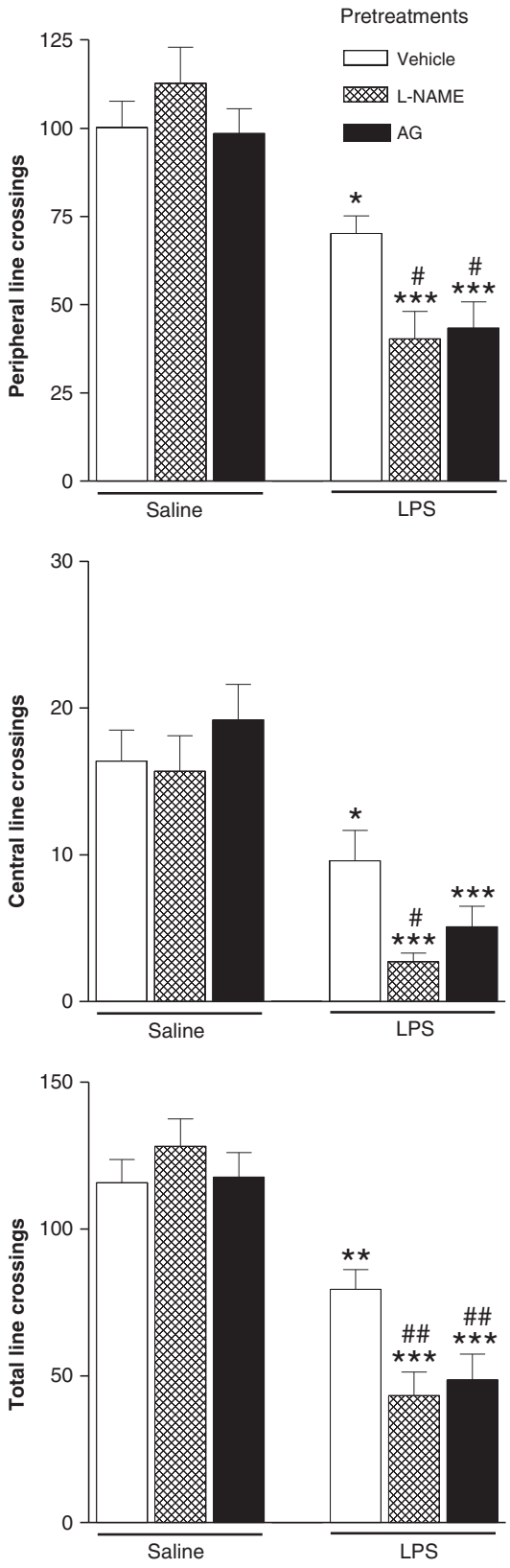

Fig. 2. Effects of pretreatment with vehicle, L-NAME (30 mg/kg) or aminoguanidine (AG, 50 mg/kg) on peripheral (A), central (B) and total (C) line crossings in the open field test (n = 10 per group), which were measured 2 h after administration of either LPS or saline. Each column represents the mean with S.E.M. The symbols denote significance levels: *$p<0.05$;**$p<0.01$; ***$p<0.001$ when compared with the control groups; # $p<0.05$; ## $p<0.01$ when compared with the vehicle plus LPS group.

3. Results

3.1. Activity in the forced swim test

L-NAME and aminoguanidine did not alter the immobility period in the FST in saline treated mice. There was a significant increase in the immobility period 120 min after administration of LPS in the FST ($F_{5,71} = 16.94$; $p<0.01$; Fig. 1) in mice pretreated with vehicle. The administration of LPS in mice previously treated with L-NAME or aminoguanidine caused a significantly higher immobility period in FST ($p<0.05$), in comparison with the vehicle plus LPS group.

3.2. Activity in the open field test

Fig. 2 shows the effect of LPS on number of line crossings in the open field, and the modulatory effects of NOS inhibition. Pretreatment with L-NAME or aminoguanidine did not significantly change the locomotor activity in the open field 120 min after i.p. saline injection. On the other hand, LPS significantly decreased the number of line crossings in the center ($F_{5,59} = 11.9$, $p<0.01$; Fig. 2A) and in the periphery ($F_{5,59} = 16.3$, $p<0.01$; Fig. 2B), as well as the total number of line crossings ($F_{5,59} = 20.28$, $p<0.001$; Fig. 2C). The number of line crossings in periphery and center of open field was even smaller in the mice pretreated with NOS inhibitors ($p<0.05$).

3.3. Behavior in the light–dark box test

The amount of time spent before the first transition in the black–white box as well as the number of entries into the light compartment were evaluated (Fig. 3). There were no significant differences in latency to the first transition after LPS administration (Fig. 3A). However, there was a significant difference in the number of transitions between the light and dark compartments ($F_{5,59} = 28.8$; $p<0{,}001$; Fig. 3B). The administration of LPS in mice previously treated with L-NAME or aminoguanidine caused a significant increase in latency to the first transition ($F_{5,59} = 4.01$; $p = 0{,}0036$; Fig. 3A), and an accentuated decrease in the number of transitions between the compartments, compared with the vehicle plus LPS group (Fig. 3B; $p<0.05$).

3.4. Food intake and body weight

LPS significantly decreased food intake 4 h after injection ($F_{7,79} = 3.65$; $p<0.01$; Fig. 4A), overnight food intake ($F_{7,79} = 6.466$; $p<0.001$; Fig. 4A) and body weight ($F_{7,79} = 3.842$; $p<0.01$; Fig. 4B). Furthermore, pretreatment with L-NAME or aminoguanidine significantly accentuated both hypophagic effects ($p<0.05$) and reduced body weight gain ($p<0.05$) induced by LPS.

3.5. Corticosterone plasma levels

Pretreatment with L-NAME or aminoguanidine did not significantly change the corticosterone plasma levels 2 h after i.p. saline injection; however, LPS injections resulted in an increase in the corticosterone plasma levels at this time. The administration of LPS in mice previously treated with L-NAME or aminoguanidine caused a significant increase in corticosterone secretion, compared with the control group (Fig. 5).

4. Discussion

The present study confirms previous observations that LPS can induce sickness behavior. These findings also provide evidence that NO acts as modulator for the development of depressive-like and exploratory behaviors in mice because non-selective NOS inhibition or selective inhibition of iNOS accentuated LPS-induced the behavioral changes.

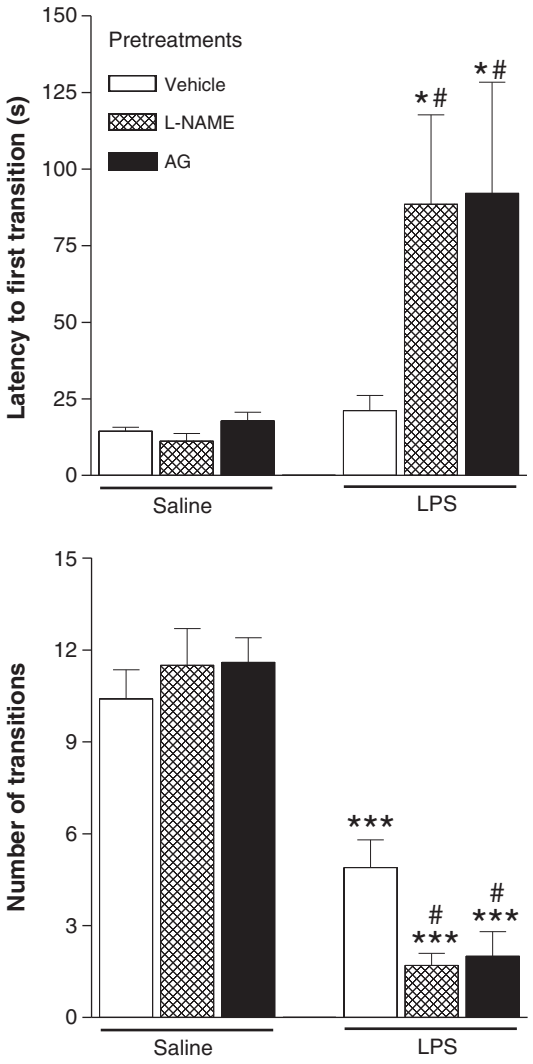

Fig. 3. Effects of pretreatment with vehicle, L-NAME (30 mg/kg) or aminoguanidine (AG, 50 mg/kg) on latency to the first transition (A) and number of transitions (B) in the light–dark box test (n = 10 per group), which were measured 2 h after administration of either LPS or saline. Each column represents the mean with S.E.M. The symbols denote the significance levels: $^{*}p<0.05$; $^{***}p<0.001$ when compared with the control groups; # $p<0.05$ when compared with the vehicle plus LPS group.

In these experiments, LPS administration increased the time spent floating in the FST and depressed the locomotor activity in the open field. These effects are characteristic of treatments that have been shown to induce depression-like behavior, and an accompanying deficit in locomotor activity would most likely affect performance in the FTS (Dunn and Swiergiel, 2005; de Paiva et al., 2010). In addition to promoting illness, LPS elicited an anxiogenic-like response in the light–dark box test. Indeed, treatment with LPS decreased the total number of transitions between dark and light compartments of the apparatus, which is a pattern believed to reflect anxiety. The proposition that LPS elicits anxiety is consistent with these findings that LPS induced an increase in corticotrophin-releasing factor (CRF) secretion, a hormone known to promote anxiety and activation of hypothalamus-pituitary-adrenal axis, as evidenced by increased corticosterone levels (Turnbull and Rivier, 1999; Rorato et al., 2009; Benedetti et al., 2011).

Activation of the immune system in response to LPS produced neural, neuroendocrine, and behavioral effects. These effects are mediated by circulating cytokines that influence brain activity by inducing the expression of neuronal NOS (nNOS) and inducible NOS (iNOS) in the hypothalamus, and the expression of cyclooxygenase (COX)-2 and microsomal prostaglandin E synthase-1 (mPGES-1) in brain vascular cells (Engblom et al., 2002, 2003; Parnet et al., 2002). Previous work has indicated that fever is mediated by increased production of prostaglandin E_2 (PGE_2) that is synthesized along the blood–brain barrier (Engblom et al., 2003), which activates a population of target neurons in the thermoregulatory center of the brain with specific PGE_2 receptors (Ushikubi et al., 1998). In addition, sickness behavior in response to LPS has been shown to be absent or greatly attenuated in mice pre-treated with inhibitors of cyclooxygenase, thereby suggesting that sickness behavior induced by LPS is mainly prostaglandin dependent and is most likely mediated by a mechanism similar to febrile response mechanisms (Ushikubi et al., 1998; Dallaporta et al., 2007; de Paiva et al., 2010). At the behavioral level, sickness behavior is a motivational state that reorganizes an organism's priorities to cope with infectious pathogens; however, the mechanisms underlying sickness behaviors have not been fully elucidated (Dantzer, 2009; Kelley et al., 2003).

LPS is a potent inducer of iNOS. Large amounts of NO and peroxynitrite, among other factors, are responsible for the hypotension, vasoplegia, apoptosis, lactic acidosis and multi-organ failure observed in the late phases of endotoxic shock. The data supporting both a detrimental and beneficial role of NO in septic shock have changed the scientific impression of this molecule. Indeed, experimental and clinical use of NOS inhibitors, which do not differentiate clearly between constitutive endothelial NOS and iNOS, prevents LPS-induced hypotension (Giusti-Paiva et al., 2002, 2004; Vona-Davis et al., 2002). However, many detrimental effects of NOS inhibitors are also reported, including increases in pulmonary resistance, decreases in cardiac output and organ perfusion, and even an increase in mortality in experimental animals (Boyle et al., 2000; Metcalf et al., 2002; Vona-Davis et al., 2002). For this reason, selective iNOS inhibitors, such as aminoguanidine or thiourea derivatives, might be preferred over nonselective NOS inhibitors for the treatment of septic shock (Cauwels, 2007). However, because iNOS-derived NO is involved in multiple pathways, selective iNOS inhibition may be not as beneficial as expected. On the other hand, previous studies have demonstrated a relation between functional protection of inhaled NO and reduced leukocyte adhesion and emigration in inflammatory lung injury (Guidot et al., 1995; Trzeciak et al., 2008). Concurrently, we found that the preservation of oxygenation in animals given NO inhalation was associated with less pulmonary sequestration of leukocytes. Accordingly, inhalation of NO gas or NO-donors in septic shock might be a complementary treatment to the use of NOS inhibitors.

In addition to the constitutive isoforms nNOS, iNOS expression has also been detected in the brain during immune challenge. iNOS is preferentially expressed in macrophages and is up-regulated in response to a variety of inflammatory stimuli; however, iNOS has been reported in the brain following endotoxin administration (Minc-Golomb et al., 1994; Harada et al., 1999). Harada et al. (1999) have demonstrated that LPS activates both nNOS and iNOS gene expression and activity in the hypothalamus, with a delayed time-course expression of iNOS compared with nNOS. Overall, there is a profound increase in NO production derived from both nNOS and iNOS in the brain after systemic inflammation or sepsis (Minc-Golomb et al., 1994; Harada et al., 1999). Despite an increase in NO production, including in the brain, during endotoxemia in mice and because NOS inhibitors crosses the blood–brain barrier (Uribe et al., 1999; Mahar Doan et al., 2000), does NO can play a role in the central mechanisms the involvement of sickness behavior induced by LPS.

Previous reports suggest that one of the roles of endogenous NO might be to restrain activation of the hypothalamic-pituitary-adrenal (HPA) axis during periods of increased cytokine and/or neuropeptide productivity, such as during immune stimulation (Uribe et al., 1999;

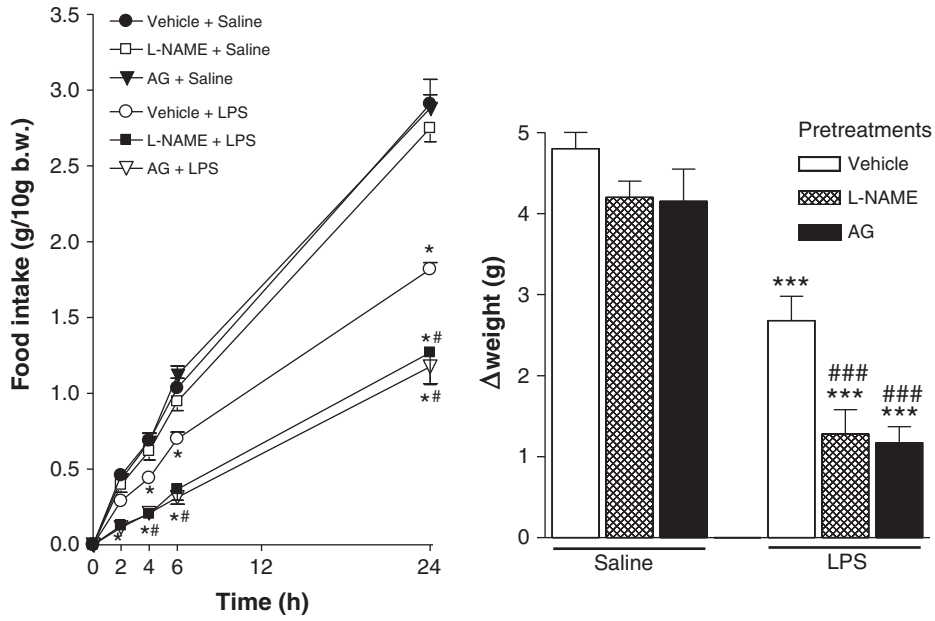

Fig. 4. Effects of pretreatment with vehicle, L-NAME (30 mg/kg) or aminoguanidine (AG, 50 mg/kg) on food intake (A), and body weight change (B) evaluated at 24 h after administration of either LPS or saline. Each column represents the mean with S.E.M. The symbols denote significance levels: $^{*}p<0.05$; when compared with the control groups; # $p<0.05$; when compared with the vehicle plus LPS group.

Bugajski et al., 2006; Gadek-Michalska and Bugajski, 2008; Jankord et al., 2009). Glucocorticoids have been suggested to play an important role in organisms coping with unpredictable events. During such events, glucocorticoids function to maintain allostasis or physiological stability (McEwen and Wingfield, 2003; Goymann and Wingfield, 2004; Romero et al., 2009). In our study, animals treated with nitric oxide synthase inhibitors prior to endotoxin administration displayed an increase in corticosterone secretion and accentuated depression-like behavior, anxiety and hypophagia. This finding suggests that NO is involved in the development of sickness behavior induced by LPS.

In conclusion, using a mouse model for acute systemic inflammation, we have shown that pharmacologically blocking NOS results in accentuated LPS-induced behavioral changes. In addition, the present study suggests that NO may have a protective role, acting in an inhibitory feedback manner, to limit LPS-induced sickness behavior.

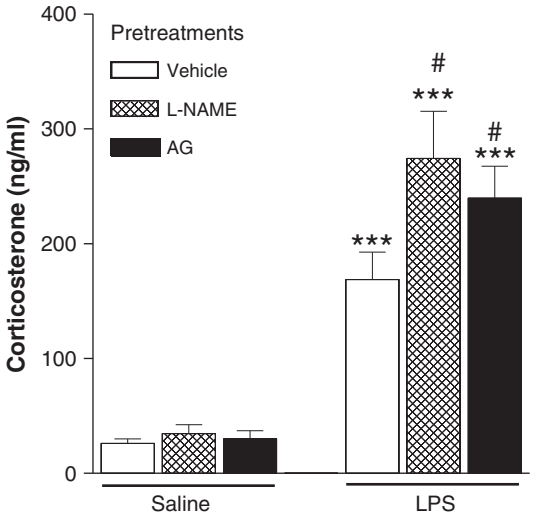

Fig. 5. Effects of pretreatment with vehicle, L-NAME (30 mg/kg) or aminoguanidine (AG, 50 mg/kg) on corticosterone plasma levels after administration of either LPS or saline. Each column represents the mean with S.E.M. The symbols denote significance levels: $^{*}p<0.05$; $^{***}p<0.001$ when compared with the control groups; # $p<0.05$; ### $p<0.001$ when compared with the vehicle plus LPS group.

Disclosures

The authors indicate no potential conflicts of interest.

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